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European Journal of Pharmacology 516 (2005) 151 – 157

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Reduced development of experimental periodontitis by treatment with M40403, a superoxide dismutase mimetic

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Received 18 November 2004; received in revised form 20 April 2005; accepted 22 April 2005
Available online 25 May 2005

Abstract

M40403, [manganese(II)dichloro[(4R,9R,14R,19R)-3,10,13,20,26 pentaazatetracyclo[20.3.1.0.(4,9)0(14,19)]hexacosa-1(26),-22(23),24-triene]], is a low-molecular-weight, synthetic, manganese-containing superoxide dismutase mimetic that removes superoxide anions without interfering with other reactive species known to be involved in inflammatory responses (e.g., nitric oxide, NO and peroxynitrite, $ONOO^-$). As such, M40403 represents an important pharmacological tool to dissect the roles of superoxide anion in acute and chronic inflammation. For this purpose, the pharmacological profile of M40403 was evaluated in a rat model of periodontitis. Periodontitis was induced in rats by placing a 2/0 braided silk around the lower left first molar. On day 8 the gingivomucosal tissue encircling the first molar was removed for biochemical and histological analysis. Ligation significantly increased inducible nitric oxide synthase activity and expression, and gingival tissue revealed increased neutrophil infiltration, lipid peroxidation and positive staining for nitrotyrosine formation and poly (ADP-ribose) polymerase activation. Ligation significantly increased Evans blue extravasation in gingivomucosal tissue and alveolar bone destruction. Intraperitoneal injection of M40403 (10 mg/kg daily for 8 days) significantly decreased all of the above-described markers of inflammation. This suggests compounds that inhibit the generation of superoxide anion, such as M40403 may be potentially useful for the treatment of periodontitis.

Keywords: Free radical; Alveolar bone loss; Reactive oxygen specie; Periodontal disease

1. Introduction

Human periodontal diseases are inflammatory disorders that give rise to tissue damage and loss as a result of complex interactions between pathogenic bacteria and the host's immune response. It is likely that the role of reactive oxygen species is common to both bacterial and host-mediated pathways of tissue damage. The formation of reactive oxygen species has been demonstrated to play an important role in the pathogenesis of a large number of pathological conditions (Halliwell, 1991) such us rheumatoid arthritis (Mccord, 1974), acute respiratory distress syndrome (ARDS: Tate and Repine, 1983), AIDS (Droge et al., 1988), and more recently periodontal disease (Kimura et al., 1993). Reactive oxygen species, such as the superoxide and hydroxyl radical, are integral reaction products of normal cellular metabolism. Aggressive periodontitis is an inflammatory disease accompanied by severe periodontal destruction, occurring in the early 20s, teens or before. Some studies have evaluated polymorphonuclear cell function in patients affected by early-onset periodontitis, but the results are discordant. Some authors reported a defective chemotactic response to formyl-met-leu-phe (Suzuki et al., 1984) and to complement-derived C5a (Genco et al., 1986), whereas others reported the chemotaxis of polymorphonuclear cells

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doi:10.1016/j.ejphar.2005.04.039

to be normal or increased in early-onset periodontitis (Repo et al., 1990). Recently, Garrett et al. (1990) demonstrated that reactive oxygen species, and particularly superoxide, are also intermediate species in the activation of osteoclasts at the site of bone resorption. Superoxide has been localized at the ruffled border space of osteoclasts, suggesting that it may be involved in bone matrix degradation (Key et al., 1994; Chapple, 1997). In various pathological situations, the use of native superoxide dismutase enzymes both pre-clinically and clinically has shed light on the importance of O_2^- in disease and, thus, the therapeutic potential of exogenous superoxide dismutase enzymes (Flohe, 1988; Huber et al., 1980; Uematsu et al., 1994). However, the native superoxide dismutase enzyme has not been evaluated in hyperglycaemia-induced electrophysiological alterations. Thus, the role of superoxide in this condition has not been defined. There are drawbacks or problematic issues associated with the use of native enzymes as therapeutic agents (e.g., solution instability, immunogenicity of non-human enzymes, bell-shaped dose–response curves, high susceptibility to proteolytic digestion) and as pharmacological tools (e.g., they do not penetrate cells or cross the blood–brain barrier, limiting the dismutation of superoxide to the extracellular space or compartment). To overcome the limitations associated with native enzyme therapy, we have developed a series of superoxide dismutase mimetics that catalytically remove O_2^-. M40403, [manganese(II)dichloro[(4R,9R,14R,19R)-3,10,13,20,26 pentaazatetracyclo[20.3.1.0.(4,9)0(14,19)]hexacosa-1(26),-22(23),24-triene]], is a prototypic example of a stable, low-molecular-weight, manganese-containing, non-peptidic molecule with the catalytic properties of the native superoxide dismutase enzymes but with the advantage of being a much smaller molecule (Salvemini et al., 1999). An important property of these superoxide dismutase mimetics is that they catalytically remove superoxide at a high rate without interacting with other biologically important reactive species, including nitric oxide, peroxynitrite, hydrogen peroxide, oxygen or hydroxyl radicals (Riley et al., 1996, 1997). This property is not shared by other classes of superoxide dismutase mimetics or scavengers, including several metalloporphyrins such as tetrakis-(*N*-ethyl-2-pyridyl) porphyrin and tetrakis-(benzoic acid)porphyrin, which interact with other reactive species, such as nitric oxide and peroxynitrite, that play important roles in inflammation (Patel and Day, 1999). We have recently demonstrated that selective removal of superoxide by superoxide dismutase mimetics such as M40403 or M40401 exerts beneficial effects in models of ischaemia and reperfusion of the intestine as well as in models of chronic inflammation (Cuzzocrea et al., 2001a). Thus, superoxide dismutase mimetics can serve as selective probes for deciphering the role of superoxide in biological systems when other relevant biological oxidants may be present. The aim of the study was to evaluate the role(s) of superoxide in a rat experimental model of periodontitis and to highlight potential mechanism(s) through which M40403 exerts its protective effects.

2. Methods

2.1. Surgical procedure

Male Sprague–Dawley rats (280–400 g) were lightly anaesthetized with surgical doses of sodium pentobarbitone (35 mg/kg). Sterile, 2/0 black braided silk thread was placed around the cervix of the lower left first molar and knotted mesially as previously described (Gyorfi et al., 1994; Di Paola et al., 2004). After the rats had recovered from the anaesthetic, they were allowed to eat commercial laboratory food and drink tap water ad libitum. Animal care and the experimental protocol were in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with EEC regulations (O.J. of E.C. L 358/1 12/18/1986). The study protocol was approved by the Institutional Animal Care and User Committee of the University of Messina.

2.2. Experimental groups

Rats were randomly allocated to the following groups: *Ligature+vehicle group*: rats were subjected to ligature-induced periodontitis and received vehicle intraperitoneally (i.p.; daily treatment for 8 days). *Ligature+M40403 group*: rats were subjected to ligature-induced periodontitis and received M40403 (10 mg/kg i.p., daily for 8 days). At different time points the rats ($N=10$ from each group for each parameter) were killed in order to evaluate the various parameters described below. The dose of M40403 used in the present studies was taken from previous studies showing its dose-dependent efficacy without side effects in models of chronic inflammation (Salvemini et al., 2001).

2.3. Measurement of arterial blood pressure indirectly in conscious rats

Mean arterial blood pressure in conscious rats was measured with a Blood Pressure Recorde (UGO BASILE, Biological Research Apparatus, 21025 Comerio, Italy). After 1 week, rats were treated as described above and blood pressure was measured before and after treatments (30 min after the last administration). To measure arterial blood pressure, rats were housed for 30 min in a warmed room (28–30 °C) and then a tail cuff was placed about 2 cm from the base of the tail and arterial blood pressure was measured. Heart rate was detected by a pulse rate counter placed after the tail cuff.

2.4. Measurement of vascular permeability by Evans blue extravasation

Vascular permeability was determined as previously described (Gyorfi et al., 1994). Extravasated Evans blue in the excised gingivomucosal tissue samples was extracted with 1 ml formamide for 48 h at room temperature for spectrophotometric determination at 620 nm. The Evans blue content is expressed as μg/g gingivomucosal tissue (Gyorfi et al., 1994).

2.5. Measurement of alveolar bone loss

In the same set of experiments, the distance from the cemento-enamel junction of the first lower molars to the alveolar crest was measured with a modification of the method of Crawford et al. (1978). Recordings were made along the median axis of the lingual surface of the mesial and mediolingual roots of the lower first left and right molars as previously described (Di Paola et al., 2004). These measurements were performed by an independent investigator who was unaware of the treatment regimens. The alveolar bone loss induced by the ligature is expressed as the difference between the left and the right sides.

2.6. Histological examination

For histopathological examination, biopsies of gingivomucosal tissue were taken 8 days after induction of ligature-induced periodontitis. The tissue slices were fixed in 10% neutral-buffered formaldehyde for 5 days, embedded in paraffin, and sectioned. The sections were stained with haematoxylin and eosin. The total number of infiltrating leukocytes (e.g., neutrophils and mononuclear cells) in cortical interstitial spaces was assessed quantitatively by counting the number of polymorphonuclear cells in 20 high-power fields.

2.7. Radiography

Mandibles were placed on a radiographic box at a distance of 90 cm from the X-ray source. Radiographic analysis of normal and ligated mandibles was performed by X-ray machine (Philips X12 Germany) with a 40-kW exposure for 0.01 s. Radiographic examination of 8 days after ligature placement revealed bone matrix resorption in the ligated lower first left molar as previously described (Di Paola et al., 2004).

2.8. Myeloperoxidase activity

Myeloperoxidase activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described (Mullane et al., 1985). At the specified time, gingivomucosal tissue was obtained and weighed. Each piece was homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000×*g* at 4 °C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. Myeloperoxidase activity stet defined as the quantity of enzyme degrading 1 μmol/min of peroxide at 37 °C and is expressed in milliunits/g of wet tissue.

2.9. Malondialdehyde measurement

Malondialdehyde levels in gingivomucosal tissue were determined as an indicator of lipid peroxidation as previously described (Ohkawa et al., 1979). Lung tissue collected at the specified times was homogenized in 1.15% (w/v) KCl solution. A 100-μl aliquot of the homogenate was added to a reaction mixture containing 200 μl of 8.1% (w/v) sodium dodecyl sulphate, 1.5 ml of 20% (v/v) acetic acid (pH 3.5), 1.5 ml of 0.8% (w/v) thiobarbituric acid and 700 μl H_2O. Samples were then boiled for 1 h at 95 °C and centrifuged at 3000×*g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

2.10. Immunohistochemical localization of nitrotyrosine and poly (ADP-ribose) polymerase

Tyrosine nitration, a specific "footprint" of peroxynitrite formation, and evidence of poly(ADP-ribose) (PAR) formation (an indicator of poly (ADP-ribose) polymerase activation) were detected as previously described (Di Paola et al., 2004) in gingivomucosal tissue sections by immunohistochemistry. At the end of the experiment, tissues were fixed in 10% (w/v) phosphate-buffered saline-(PBS)-formaldehyde and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) H_2O_2 in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the sections in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA, Milan, Italy). The sections were then incubated overnight with primary anti-nitrotyrosine antibody (1:1000 dilution), primary anti-PAR (1:500 dilution) with control solutions including buffer alone or non-specific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). Immunocytochemistry photographs ($n=5$ from each sample collected from all rats in each experimental group) were assessed by densitometry analysis, using Optilab Graftek software on a Macintosh personal computer.

2.11. Materials

M40403 was kindly supplied by Dr. Daniela Salvemini, Department of Biological and Pharmacological Research, Meta-Phore Pharmaceuticals, 1910 Innerbelt Business Center Drive, St. Louis, Missouri 63114, USA. Primary anti-nitrotyrosine antibody was from Upstate Biotech (DBA, Milan, Italy). All other reagents and compounds used were obtained from Sigma Chemical Company (Sigma, Milan, Italy).

2.11.1. Data analysis

All values in the figures and text are expressed as means ± standard error of the mean of *n* observations, where *n* represents the number of animals studied. Data sets were examined by one- and two-way analysis of variance and individual group means were then compared with Bonferroni or Student's unpaired *t*-test. A *P*-value less than 0.05 was considered significant. In the experiments involving histology or immunohistochemistry, the data are representative of at least 3 experiments performed on different experimental days.

3. Results

3.1. Effects of M40403 on neutrophils infiltration and lipid peroxidation in periodontitis

Myeloperoxidase activity was significantly elevated ($P<0.001$) at 8 days after ligation (Fig. 1A) and M40403-treatment

significantly reduced these levels. Malondialdehyde levels, indicative of lipid peroxidation, were significantly increased in the gingivomucosal tissues of rats with ligature-induced periodontitis (Fig. 1B). Gingivomucosal tissues malondialdehyde levels were significantly reduced in rats treated with M40403 (Fig. 1B). No significant changes in either myeloperoxidase activity or malondialdehyde levels were observed in the gingivomucosal tissues from the controlateral side (Fig. 1A, B).

3.2. Effect of M40403 on plasma extravasation and bone destruction

Before the measurement of Evans blue extravasation, the mean arterial pressure of vehicle-treated and M40403-treated animals was recorded. In agreement with previous studies (Cuzzocrea et al., 2004), M40403 treatment did not affect mean arterial blood pressure (vehicle-treated: 116+6 mm Hg; $n=10$ and M40403-treated: 105+7 mm Hg; $n=10$). After Evans blue injection, in contrast to the controlateral side, in the gingiva around the neck of the teeth a definite blue belt could be observed even in non-ligated control animals (not shown). Ligation significantly increased Evans blue extravasation in gingivomucosal tissue compared to that on the contralateral side (Fig. 1C). M40403 treatment prevented this increase in Evans blue extravasation, but did not change the Evans blue content of the contralateral side (Fig. 1C).

A radiographic examination of the mandibles at 8 days after ligature placement revealed bone matrix resorption in the lower left first molar region (Fig. 2A). There was no evidence of pathology in the right first molar (data not shown). M40403 markedly reduced the degree of bone resorption in the lower left first molar region after ligation (Fig. 2B). In addition, significant alveolar bone loss between the lower first left and the right molars induced by ligation was observed in vehicle-treated rats (Fig. 2C). M40403 treatment resulted in a significant inhibition of alveolar bone loss after ligation (Fig. 2C).

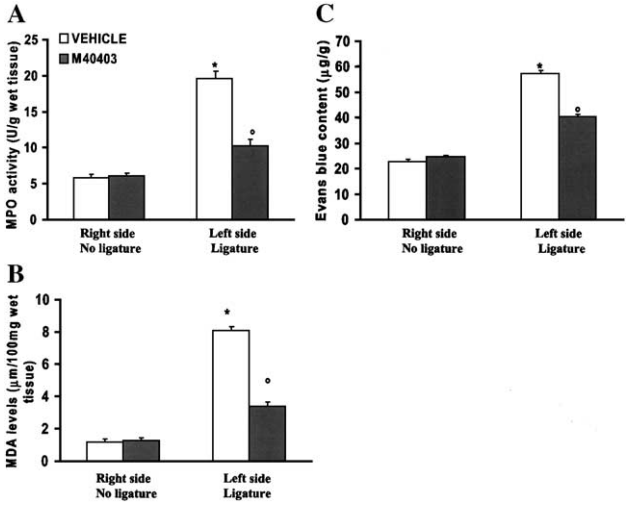

Fig. 1. Myeloperoxidase activity (A), malondialdehyde levels (B) and Evans blue content (C) in gingivomucosal tissue was significantly increased by ligation compared to those on the contralateral non-ligated side. M40403 (10 mg/kg i.p., daily for 8 days) significantly reduced myeloperoxidase activity, malondialdehyde levels and Evans blue content. Data are means±S.E.M. from $n=10$ rats for each group. *$P<0.01$ vs. non-ligated. °$P<0.01$ vs. ligated.

3.3. Effects of M40403 on nitrotyrosine and poly (ADP-ribose) polymerase

Sections of gingivomucosal tissue from the contralateral side did not reveal any immunoreactivity for nitrotyrosine (Figs. 2D and 3A) or for anti-poly (ADP-Ribose) polymerase (Figs. 2D and 3D) within the normal architecture. Eight days following ligation, positive staining for nitrotyrosine (Figs. 2D and 3B) and for anti-poly (ADP-Ribose) polymerase (Figs. 2D and 3E) was found in the gingivomucosal tissue from rats with ligated molars. M40403 (10 mg/kg, i.p.) reduced the staining for both nitrotyrosine and anti-poly (ADP-Ribose) polymerase (Figs. 2D and 3C, F respectively).

3.4. Effects of M40403 on ligature-induced periodontitis

When compared to gingivomucosal tissue sections taken from the controlateral side (Fig. 4A), gingivomucosal tissue sections from rats with ligated molars showed oedema, tissue injury and infiltration of the tissue by inflammatory cells (Fig. 4B). M40403 treatment reduced the degree of gingivomucosal tissue injury (Fig. 4C). Quantification of infiltrating polymorphonuclear cells into gingivomucosal tissue showed that there was only a minimal number of polymorphonuclear cells in tissue from the controlateral side (Fig. 4A and D). However, a large number of infiltrating polymorphonuclear cells were observed in the gingivomucosal tissue of ligated rats (Fig. 4B and D). M40403 administration significantly reduced the number of polymorphonuclear cells infiltrating into gingivomucosal tissue by approximately 70% (Fig. 3C and D).

4. Discussion

In the present study, a well-established rat model of acute periodontitis was used, which involves placing a ligature around the cervix of the mandibular first molar tooth. A similar model has previously been used in several species (Schroeder and Lindhe, 1975). This study suggests a potential therapeutic application of M40403, a new superoxide dismutase mimetic, for the treatment of inflammatory periodontal disease. In particular, we demonstrated that M40403 reduced (i) the development of ligature-induced periodontitis, (ii) the infiltration of gingivomucosal tissue by polymorphonuclear cells, (iii) lipid peroxidation in gingivomucosal tissue and (iv) gingivomucosal tissue injury (histology) in rats subjected to ligature-induced periodontitis. All of these findings support the view that the superoxide anion plays an important role in this model of periodontitis.

Some important pro-inflammatory roles for superoxide anion include: endothelial cell damage and increased microvascular permeability; formation of chemotactic factors, such as leukotriene B_4; recruitment of neutrophils at sites of inflammation; lipid peroxidation and oxidation; DNA single-strand damage; and formation of peroxynitrite

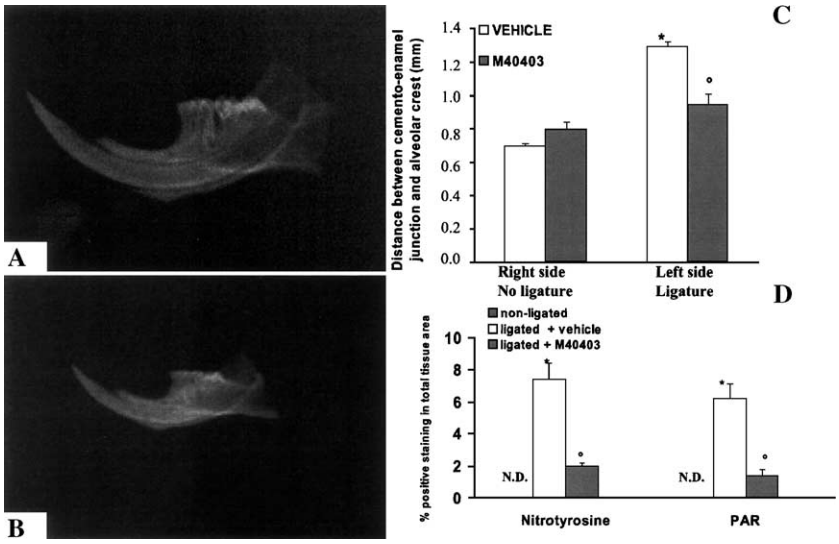

Fig. 2. The alveolar bone from ligated (8 days) rats demonstrated alveolar bone resorption (A). M40403 treatment suppressed alveolar pathology in the rat alveolar bone (B). A significant increase in the distance between the cemento-enamel injunction and the alveolar crest at the mediolingual root of the first molar was observed in molar-ligated rats. M40403 treatment significantly reduced the increase in the distance between the cemento-enamel injunction and the alveolar crest. Densitometry analysis of immunocytochemistry photographs (D; $n=5$ photos from each sample collected from all rats in each experimental group) for poly (ADP-ribose) (PAR) and nitrotyrosine in gingivomucosal tissue. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). The radiographic data are representative of at least 3 experiments performed on different experimental days. Densitometry data are expressed as % of total tissue area. Data represent the data from 20 counts obtained from gingivomucosal tissue of each treatment group. *$P<0.01$ vs. non-ligated. °$P<0.01$ vs. ligated.

(Cuzzocrea et al., 2001b). Our results demonstrated that M40403 exerted a significant inhibitory effect on plasma extravasation and reduced the extent of bone resorption during periodontitis. Our study also confirmed earlier findings that one of the characteristic signs of inflammation, Evans blue extravasation, was higher on the ligated side on the 8th day than on the opposite side (Gyorfi et al., 1994). The hypothesis that highly reactive radicals may be responsible for the initial degradation of extracellular matrix components seen in periodontal disease is in

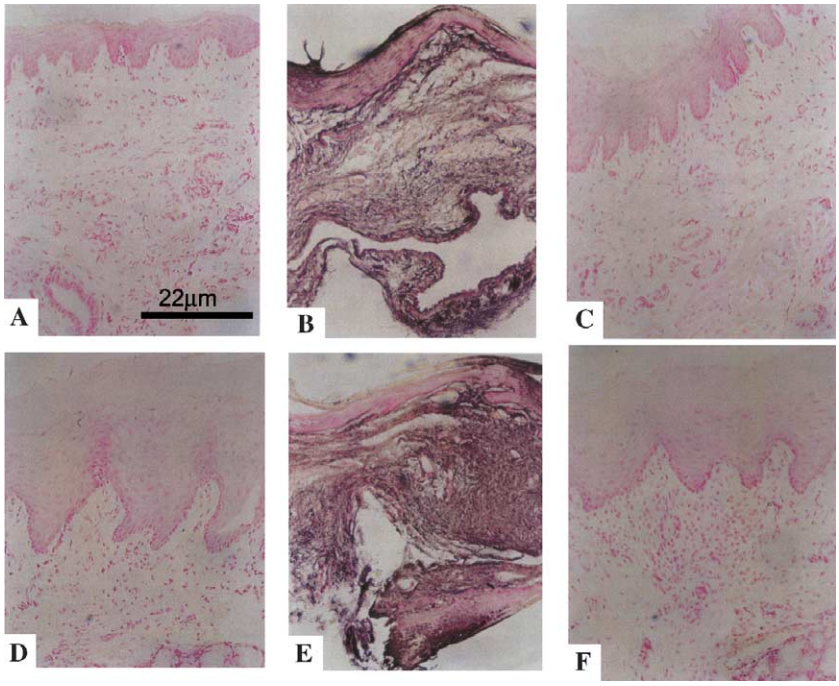

Fig. 3. Immunohistochemical staining for nitrotyrosine and poly (ADP-Ribose) polymerase formation. No staining for nitrotyrosine (A) and poly (ADP-Ribose) polymerase (D) was observed in control gingivomucosal tissue while positive staining was observed after ligation (B and E). In gingivomucosal tissue from M40403 (10 mg/kg i.p., daily for 8 days)-treated rats, no positive staining was observed for either nitrotyrosine (C) or poly (ADP-Ribose) polymerase (F). Original magnification: ×125. The data are representative of at least 3 experiments performed on different experimental days.

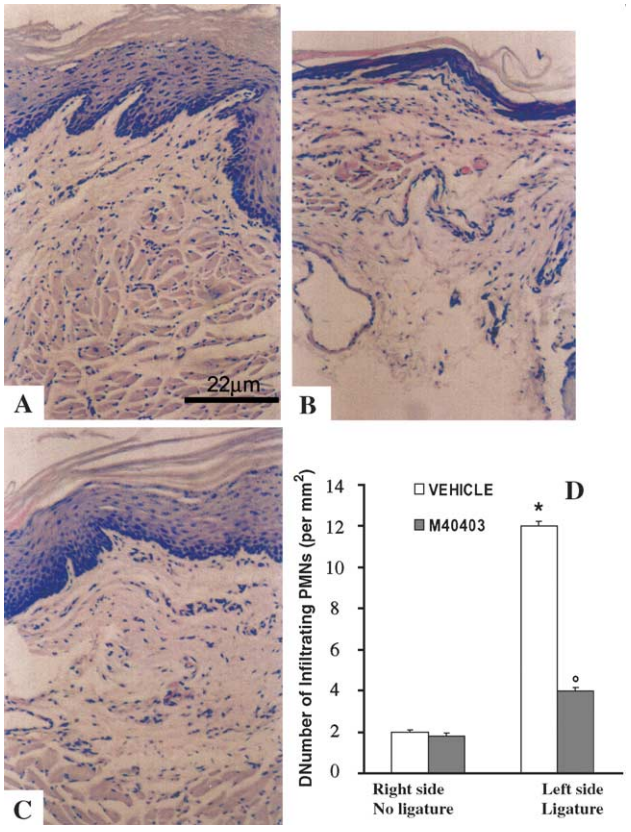

Fig. 4. Gingivomucosal section from control (non-molar ligated) rats (A) demonstrating no tissue damage. Inflammatory cells infiltration and oedema was observed in gingivomucosal section from molar-ligated rats (B). Significantly less oedema and inflammatory cell infiltration was observed in gingivomucosal sections from molar-ligated rats treated with M40403 (10 mg/kg i.p., daily for 8 days) (C). The total number of infiltrating leukocytes (e.g., neutrophils and mononuclear cells) in gingivomucosal tissue was assessed quantitatively by counting the number of polymorphonuclear cells in 20 high-power fields (D). Original magnification: ×125. Data are representative of at least 3 experiments performed on different experimental days. Data represent the means±S.E.M. for 20 counts obtained from gingivomucosal tissue from each treatment group. *$P<0.01$ vs. non-ligated. °$P<0.01$ vs. ligated.

agreement with the findings of Misaki et al. (1990), who demonstrated improved healing of gingival wounds in rats after i.v. application of superoxide dismutase. The enzyme superoxide dismutase also prevents amplification and interrupts the cascade of free radical formation, i.e. single superoxide molecules interact with lipids, H_2O_2, flavins to form other radicals (Michelson et al., 1986). We report in the present study that ligature-induced periodontitis in the rat results in a significant infiltration of inflammatory cells into gingivomucosal tissues. In previous studies, superoxide was found to increase both neutrophil infiltration and adhesion (Cuzzocrea et al., 2001a). We also demonstrated in the present study that treatment with M40403 reduced this inflammatory cell infiltration, as assessed by the specific granulocyte enzyme myeloperoxidase, and moderated tissue damage, as evaluated by histological examination. A possible mechanism by which M40403 attenuates polymorphonuclear cell infiltration is the down-regulating of the adhesion molecules ICAM-1 and P-selectin, as previously demonstrated (Cuzzocrea et al., 2001a). These findings are in accordance with those of Berglundh and Lindhe (1993), who also found a significant increase in inflammatory cell infiltration in inflamed gingiva as compared to healthy gingiva. In addition, the area of infiltrated connective tissue in free gingiva was smaller when scaling and root planning was followed by M40403 administration. Furthermore, we found that the tissue damage induced by ligature in vehicle-treated rats was associated with high levels of tissue malondialdehyde, which is considered a good indicator of lipid peroxidation (Di Paola et al., 2004). Intense immunostaining for nitrotyrosine formation also suggested that a structural alteration of gingivomucosal tissues had occurred, most probably due to the formation of highly reactive nitrogen derivatives. It has been demonstrated that several chemical reactions involving nitrite, peroxynitrite, hypochlorous acid and peroxidases can induce tyrosine nitration and may contribute to tissue damage, including that of gingivomucosal tissue (Lohinai et al., 1998). In addition to nitric oxide, peroxynitrite is also generated in ligature-induced periodontitis (Lohinai et al., 1998).

Therefore, in this study we clearly demonstrated that M40403 treatment prevented the release of superoxide anion and the formation of peroxynitrite. Reactive oxygen species produce strand breaks in DNA, which trigger energy-consuming DNA repair mechanisms, and activate the nuclear enzyme poly (ADP-Ribose) polymerase. There is evidence that the activation of poly (ADP-Ribose) polymerase may also play an important role in inflammation (Szabo, 1997). We demonstrated here that M40403 treatment reduced the activation of poly (ADP-ribose) polymerase in the gingivomucosal tissue during ligature-induced periodontitis. Thus, we propose that the anti-inflammatory effects of M40403 may be, at least in part, due to the prevention of the activation of poly (ADP-ribose) polymerase. In conclusion, this study provides the first evidence that M40403 causes a substantial reduction of ligature-induced periodontitis in the rat. Finally, our findings suggest that interventions which reduce the generation or the effects of superoxide anion may be useful in conditions associated with local or systemic inflammation.

Acknowledgements

This study was supported by grant from PRA 2003 from University of Messina. The authors would like to thank Giovanni Pergolizzi and Carmelo La Spada for their excellent technical assistance during this study, Mrs. Caterina Cutrona for secretarial assistance and Miss Valentina Malvagni for editorial assistance with the manuscript.

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